

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY
(Leave blank)

2. REPORT DATE
May 2004

3. REPORT TYPE AND DATES COVERED

Annual Summary (1 May 2003 - 30 Apr 2004)

4. TITLE AND SUBTITLE

Novel Recombinant Fab Fragments of the Tag-72 Monoclonal Antibody CC49 Containing an Integrated Radiometal Binding Site for Radioimmunoguided Surgery for DCIS

5. FUNDING NUMBERS

DAMD17-03-1-0372

6. AUTHOR(S)

Ying Tang

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University Health Network
Toronto, Ontario M5G 1Z5
Canada

E-Mail: ying.tang@uhn.on.ca

8. PERFORMING ORGANIZATION
REPORT NUMBER

9. SPONSORING / MONITORING

AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING
AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

20041021 024

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Ductal carcinoma *in situ* (DCIS) is an early form of breast cancer. Surgical treatment of DCIS is currently inadequate due to the inability to define accurately the extent of the disease. Radioimmunoguided surgery (RIGS) employing radionuclide-conjugated monoclonal antibodies against breast cancer antigens and a sensitive γ -detecting probe may improve the surgical management of DCIS by more clearly identifying malignant tissue. TAG-72 antigen is overexpressed in 81% of DCIS by immunohistochemical staining with monoclonal antibody CC49. Our objective was to construct a novel recombinant Fab (rFab) of CC49 containing an integrated radiometal-binding site that can be directly labeled with ^{99m}Tc through the C-terminal hexahistidine (His_6) for RIGS of DCIS. In the first year of the project, I have cloned the genes of Fab (L and Fd) from CC49 hybridoma cells, constructed a co-expression plasmid to express rFab of CC49 in *Pichia pastoris*, and purified the rFab to homogeneity. This rFab was immunoreactive to TAG-72 *in vitro*; rFab labeled with ^{123}I localized specifically in TAG-72 positive s.c. LS174T cancer xenografts in athymic mice. In the second year, rFab will be labeled with ^{99m}Tc through its incorporated His_6 . The tumor targeting properties of ^{99m}Tc -rFab will also be evaluated in the xenograft model.

14. SUBJECT TERMS

Breast cancer, DCIS, Tc-99m, CC49, recombinant antibodies, Fab

15. NUMBER OF PAGES

15

16. PRICE CODE

17. SECURITY CLASSIFICATION
OF REPORT

Unclassified

18. SECURITY CLASSIFICATION
OF THIS PAGE

Unclassified

19. SECURITY CLASSIFICATION
OF ABSTRACT

Unclassified

20. LIMITATION OF ABSTRACT

Unlimited

AD_____

Award Number: DAMD17-03-1-0372

TITLE: Novel Recombinant Fab Fragments of the Tag-72 Monoclonal Antibody CC49 Containing an Integrated Radiometal Binding Site for Radioimmunoguided Surgery for DCIS

PRINCIPAL INVESTIGATOR: Ying Tang

CONTRACTING ORGANIZATION: University Health Network
Toronto, Ontario M5G 1Z5
Canada

REPORT DATE: May 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

TABLE OF CONTENTS

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Summary of Research Accomplished in Year 1 (2003-2004).....	4
<i>Task 1: Clone the genes of CC49 Fab and express recombinant Fab fragments of CC49 in Pichia pastoris and purify the recombinant Fab fragments</i>	4
<i>Task 2: Assess the immunoreactivity of the recombinant Fab</i>	11
<i>Task 4: Conduct in vivo imaging and biodistribution studies in a mouse tumor xenograft model...</i>	12
Planned Research for Year 2 (2004-2005).....	13
<i>Task 2 (completion): Assess the immunoreactivity of the recombinant Fab</i>	13
<i>Task 3: Label recombinant Fab with ^{99m}Tc-tricarbonyl complex through the C-terminal His₆ tag...</i>	13
<i>Task 4 : Conduct in vivo imaging and biodistribution studies in a mouse tumor xenograft model...</i>	13
<i>Task 5: Analyze data statistically and prepare manuscripts</i>	13
Key Research Accomplishments.....	13
Reportable Outcomes.....	14
Manuscripts.....	14
Abstracts.....	14
Presentations.....	14
Conclusions.....	14
References.....	15

INTRODUCTION

Ductal carcinoma *in situ* (DCIS) is an early form of breast cancer in which cancer cells are confined by the basement membrane in the ducts. Although the mortality associated with DCIS at diagnosis is negligible, the importance of treating DCIS is that the disease is a precursor of most invasive breast cancer. Moreover, the incidence of DCIS (1) has been increasing presumably due to the widespread use of mammographic screening for early detection of breast cancer (1, 2). DCIS currently accounts for up to 30% of newly diagnosed breast cancers (2). The disease is most often treated by breast conserving surgery (BCS), which may be combined with local radiation therapy (3, 4). Local recurrence after BCS, however, remains a problem. About 10 to 30% of DCIS patients treated by local excision with a histological tumor-free margin subsequently develop recurrent disease and require re-excision. Furthermore, invasive breast cancer presents in about half of the recurrent cases (5-7). Local recurrence after BCS is believed to originate from residual DCIS (8). Therefore, there is a need to improve the accuracy of delineating the extent of DCIS in order to achieve complete excision of the disease, which may reduce the rate of local recurrence as well as mortality. Radioimmunoguided surgery (RIGS) is a technique that employs a sensitive γ -detecting probe and a specific radiolabeled anti-tumor antibody. The surgeon uses the γ -probe to define more accurately the extent of tumor through identifying intraoperatively tumor tissue that is pretargeted by a radiolabeled antibody. Monoclonal antibody CC49 is a murine IgG₁ directed against tumor-associated glycoprotein-72 (TAG-72), an epithelial mucin. CC49 was found highly reactive with 81% of DCIS by immunohistochemistry (9). The objectives of the study are: 1) to generate recombinant Fab of CC49 containing an integrated hexahistidine radiometal-binding site through expression in yeast, purify the resulting Fab, and evaluate its immunoreactivity to TAG-72 *in vitro*; 2) to label recombinant Fab of CC49 with ^{99m}Tc directly through the hexahistidine moiety and evaluate the stability and immunoreactivity *in vitro*; 3) to evaluate the tumor and normal tissue localization of the radiolabeled recombinant Fab in a tumor xenograft model.

SUMMARY OF RESEARCH ACCOMPLISHED IN YEAR 1 (2003-2004)

The following represents a summary of the research accomplished in the first year of the project.

Task 1: Clone the genes of CC49 Fab and express recombinant Fab fragments of CC49 in Pichia pastoris and purify the recombinant Fab fragments

Fab fragments of an antibody consist of the light (L) chain covalently linked to the Fd ($V_H + C_H1$) portion of the heavy chain through a disulfide bond. In this study, recombinant Fab (rFab) of monoclonal antibody CC49 (Fig. 1) consists of the L chain fused with a FLAG tag (DYKDDDDK) and the Fd chain fused with a hexahistidine (His₆) tag. A flexible peptide linker (GGGGS) is inserted between the tags and each chain of Fab. The FLAG tag is designed for the detection of L; the His₆ tag is for the detection of Fd as well as purification of rFab by immobilized metal affinity chromatography (IMAC). The His₆ tag can also be used for direct labeling with complex ^{99m}Tc (10).

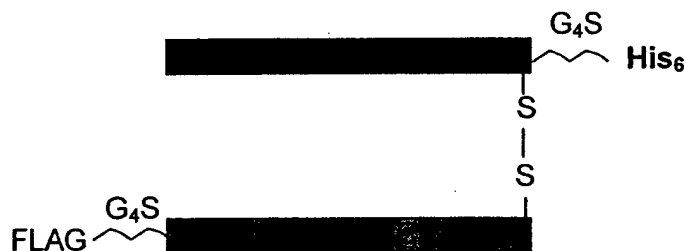


Fig. 1. Schematic representation of the construct of CC49 rFab. L consists of a variable region (V_L) and a constant region (C_L); Fd consists of a variable region (V_H) and the first constant region (C_{H1}) from the heavy chain. A His_6 tag is fused to Fd at its carboxyl terminus, and a FLAG tag is fused to L at its amino terminus. A flexible peptide linker (GGGGS or G_4S) connects the tags to L or Fd.

Cloning the genes of L and Fd (V_H+C_{H1}) of CC49 IgG from hybridoma cells into a TA cloning vector pCR[®]2.1. The CC49 hybridoma cell line producing monoclonal antibodies against TAG-72 was provided by Dr. J. Schlom in NCI (U.S.) through a Material Transfer Agreement with Dr. Reilly, my PhD dissertation mentor. The hybridoma secretes murine CC49 IgG_{1,k}. Total RNA was extracted from 1×10^6 hybridoma cells using RNeasy[®] kit (Qiagen, Valencia CA). First-strand cDNA was synthesized from the pool of total RNA using Superscript II[®] reverse-transcriptase (Invitrogen, San Diego CA) primed with random hexamer primers (Invitrogen). The target genes were amplified by PCR from cDNA using primers specific for the sequences flanking the coding regions of L (11, 12) or Fd (13, 14) (GenBank accession nos. L14549, L14553, V00802, and J00453). The forward primer H_FOR-1 and the reverse primer H_REV-1 (Table 1) were for amplification of the DNA fragment containing Fd; L_FOR-1 and L_REV-1 were for the DNA fragment containing L. The bands on the gel corresponding to DNA from L (775 bp) and H (837 bp) chains were excised. DNA was extracted from the gel using QIAquick Gel[®] extraction kit (Qiagen), and was reconstituted in 50 μL of EB buffer (10 mM Tris HCl pH 8.5).

The purified DNA fragments containing L or Fd from PCR was ligated into a pCR[®]2.1 cloning vector (Invitrogen) to transform TOP10F' cells. Transformed colonies were selected on a LB agar plate containing 50 $\mu\text{g}/\text{mL}$ of kanamycin (Invitrogen) according to the manufacturer's instruction. Plasmid DNA was extracted and purified from the transformed cells, and the inserts were sequenced with T7 (forward) and M13 reverse primers. The resulting plasmids were denoted as pCR2.1-L (containing the L insert) and pCR2.1-Fd (containing the Fd insert).

Construction of Plasmids Expressing CC49 rFab. An expression plasmid based on the *P. pastoris* secretion vector pPICZ α A was constructed to co-express L and Fd of CC49. In the co-expression plasmid (pPIC-L-Fd or pPIC-Fd-L, Fig. 2B), the expression of L and Fd is driven individually by a methanol-inducible 5'*AOX1* promoter. An α -factor secretion signal sequence from *Sacharomyces cerevisiae*, which directs the secretion of heterologous proteins into the culture medium, immediately precedes the sequences encoding L or Fd. A transcription termination sequence follows the inserted L

and Fd genes. L and Fd were expressed as separate polypeptides, but assembled into rFab in *P. pastoris* through disulfide bond formation. rFab then was secreted into the culture medium.

Table 1. Oligonucleotide primers used for constructing plasmids to co-express L and Fd

Oligonucleotide	Nucleotide Sequence ^a	annotations
Cloning L and Fd into the pCR2.1 cloning vector		
L_FOR-1	5'-TATGTTACTGCTGCTATGGGTATCTG-3'	cloning
L_REV-1	5'-CTTCCCTTCTAAGGTCTTGGAGGCTTC-3'	cloning
H_FOR-1	5'-GTCTTCTCCGCTATCCCTGGACACACTG-3'	cloning
H_REV-1	5'-CCAAGGATGTGCTCACCATTACTCTGACT-3'	cloning
Subcloning L and Fd into the pPICZαA vector		
L_FOR-2	5'-CTAGTGAATTCGACTACAAGGACGACGACACAAGGG TGGCGGTGGCTCGGACATTGTGATGTCACAGTCTCCATC-3'	<i>EcoR I</i> ; FLAG tag bold
L_REV-2	5'-CACTGTCTAGATCACTAACACTCATTCTGTTGAA GCTCTTGACAATGG-3'	<i>Xba I</i>
H_FOR-2	5'-CTAGTGAATTCGAGGTTTCAGTTGCAGCAGTCTGACGCTG AGTTG-3'	<i>EcoR I</i>
H_REV-2	5'-CACTGTCTAGATCACTAATGATGATGATGATGATGCGA GCCACGCCACCACAATCCCTGGGCACAATTTTCTTGTC-3'	<i>Xba I</i> ; His ₆ tag bold
H_FOR ΔBam	5'-GTGACCTGGAAGTCTGGTCCCTGTCCAGC-3'	<i>BamH I</i> silencing
H_REV ΔBam	5'-GCTGGACAGGGAACAGAGTTCCA GGTCAC-3'	<i>BamH I</i> silencing
Constructing plasmids to co-express L and Fd		
ΔPmeI_FOR	5'-CAAACTGACAGTTTATACGCTGTC-3'	<i>PmeI</i> mutation
ΔPmeI_REV	5'-GACAGCGTATAAACTGTCAGTTTTC-3'	<i>PmeI</i> mutation
Bgl_FOR	5'-GAGAAGATCTAACATCCAAAGACG-3'	<i>Bgl II</i>
Bam_REV1798	5'-CGACACCCTAGAGGAAGAAAGAGG-3'	

^a Restriction sites are underlined; stop codons are italicized; mismatches are in lowercase.

Primers for sequencing are based on the DNA sequences from the α-factor priming site (forward primer 5'-TATTGCCAGCATTGCTGC-3') and the 3'AOX1 priming site (reverse primer 5'-GCAAATGGCATTCTGACATCC-3') in pPICZαA.

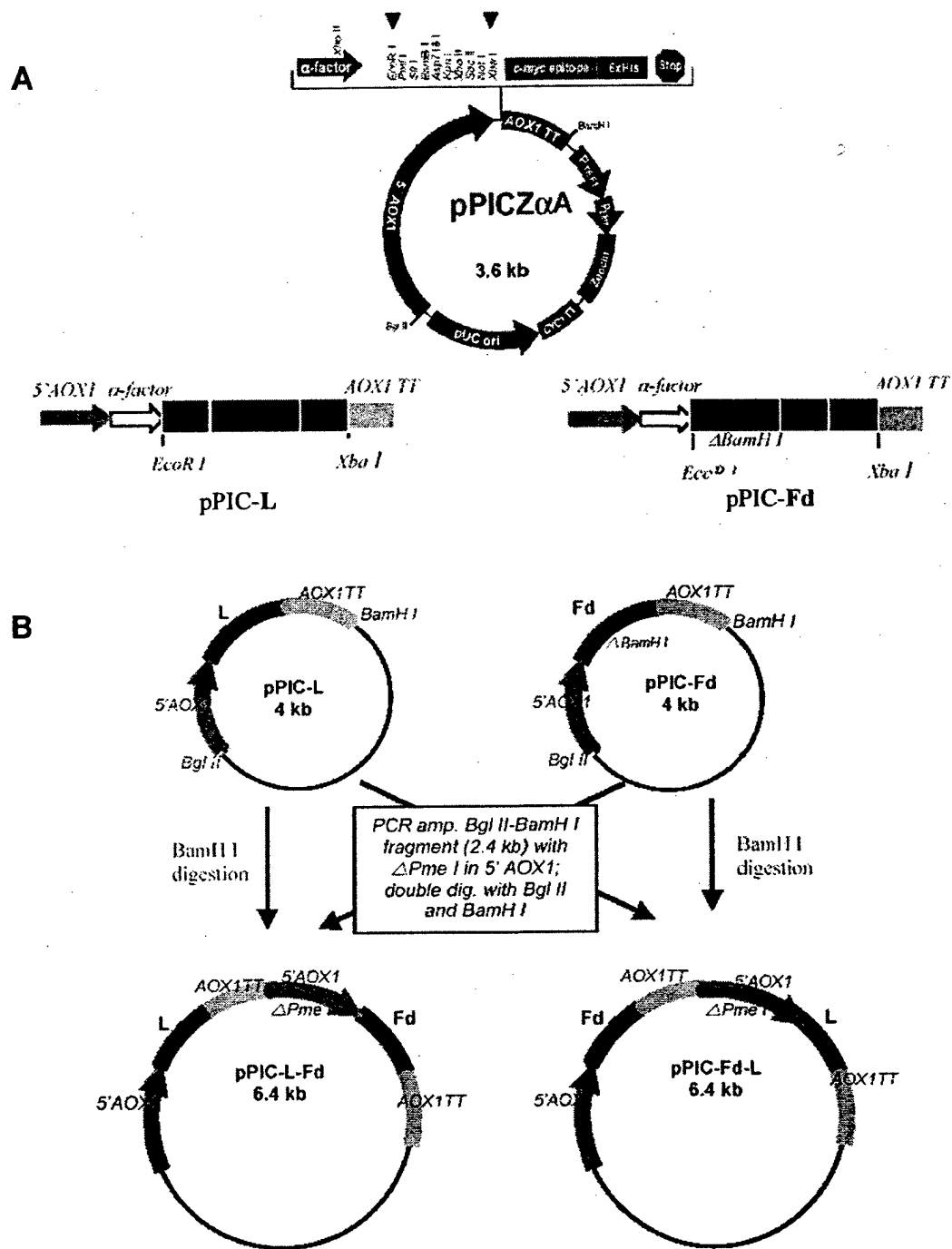


Fig. 2. Construction of co-expression plasmids for expression of rFab of CC49. (A) Map of the pPICZαA expression vector (upper) and a linear representation of the expression plasmids containing a single insert of L or Fd gene (lower). (B) The strategy to construct the co-expression plasmids pPIC-Fd-L and pPIC-L-Fd.

The co-expression plasmid was constructed in two steps (Fig. 2). In the first step (Fig. 2A), L and Fd were PCR amplified from the cloning plasmids, pCR2.1-L and pCR2.1-Fd, using primers (L_FOR-2, L_REV-2, H_FOR-2, and H-REV-2; Table 1) to install the *EcoR* I sites at the amino terminus and the *Xba* I sites at the carboxyl terminus for subsequent insertion into an expression vector pPICZαA. Also included in the primers were sequences of the FLAG tag and the His₆ tag. The FLAG tag was placed after the *EcoR* I site and fused to the amino terminus of L through a flexible GGGGS linker; the His₆ tag was fused to the carboxyl terminus of Fd through the linker. Two stop codons were installed before the *Xba* I sites. Without altering the amino acid sequence, the *Bam*H I site within Fd was disrupted by site-directed mutagenesis introduced by overlap extension PCR (primers H_FOR_ΔBam and H_REV_ΔBam). This mutation was necessary for the construction of a co-expression plasmid where *Bam*H I in the expression plasmid containing either L or Fd was used as the cloning site for inserting the second expression cassette. The PCR-amplified L and Fd genes flanked by the *EcoR* I and *Xba* I sites were digested and ligated into a pPICZαA vector individually. The ligated plasmids were then used to transform TOP10F' cells. Transformants were selected on low salt LB agar plates containing 25 μg/ml Zeocin[®] (Invitrogen). The DNA inserts in the plasmids from the transformants were sequenced and their orientation determined. The plasmids containing correctly oriented L and Fd genes were denoted as pPIC-L and pPIC-Fd respectively (Fig. 2A). In the second step (Fig. 2B), the plasmid, pPIC-L or pPIC-Fd, containing a single L or Fd gene was combined to create a co-expression plasmid that harbored both the L expression cassette and the Fd expression cassette. As illustrated in Fig. 2B, the expression cassette containing L was amplified by PCR (forward primer Bgl_FOR, reverse primer Bam_REV1798; Table 1) from pPIC-L as a *Bgl* II and *Bam*H I fragment; it was then cloned into pPIC-Fd at the *Bam*H I site immediately after the Fd expression cassette to generate the co-expression plasmid pPIC-Fd-L. Similarly, a co-expression plasmid pPIC-L-Fd was constructed by inserting PCR amplified Fd expression-cassette into pPIC-L. These two co-expression plasmids are only different in the relative position of the L and Fd expression-cassettes in the plasmids. The *Pme* I site inside 5'*AOX1* of the PCR-amplified expression cassettes was disrupted by site-directed mutagenesis using overlap extension PCR to introduce a single nucleotide mutation (primers ΔPmeI_FOR and ΔPmeI_REV) thereby ensuring that only one *Pme* I site existed in the co-expression plasmids for subsequent linearization, which was necessary for efficient integration of the construct into the genomic DNA of *P. pastoris*. TOP10F' cells were transformed with the pPIC-Fd-L or pPIC-L-Fd plasmid, and transformants were selected with 25 μg/mL Zeocin[®] on low salt LB agar plates. Plasmid DNA was isolated from positive transformants using Qiagen HiSpeed[®] Midi Kit (Qiagen). The orientation of the inserts in the co-expression plasmids was determined by PCR. The co-expression plasmids with tail-to-head oriented expression cassettes were sequenced and subsequently used for rFab expression.

Expression of CC49 rFab in *P. pastoris* in Shake Flask and rFab Purification. The pPIC-Fd-L or pPIC-L-Fd plasmids were linearized at the unique *Pme* I site in the first 5'*AOX1* promoter region before electro-transforming *P. pastoris* KM71H cells using a MicroPulser[®] electroporator (Bio-Rad, Hercules CA). The transformed KM71H cells were selected with Zeocin[®] at 100 μg/ml. In order to screen for transformants expressing the greatest amount of rFab, six Zeocin[®]-resistant transformants derived from pPIC-Fd-L or pPIC-L-Fd were tested for expression. Clone #24-5 (pPIC-Fd-L) and Clone #5-3 (pPIC-L-Fd) were identified as expressing the highest amount of rFab in BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base with ammonium sulfate without amino acids, 4 x 10⁻⁵ % biotin, 0.5% methanol) when induced with 0.5% methanol every 24 hours. rFab secreted in BMMY was measured by ELISA. ELISA was carried out using bovine submaxillary mucin (BSM, Sigma) as a source of the TAG-72 antigen (15). Briefly, a 96-well

micro-ELISA plate (Corning Costar®, Corning Inc., Corning NY) was coated with 2 µg of BSM in 100 µL of PBS pH 7.0 at 4°C overnight. Non-specific binding sites were saturated by incubating the wells with 5% BSA in PBS at 4°C overnight. The culture supernatant collected at different times up to 96 hours after methanol induction was added in duplicate to the wells (100 µL/well) and the plate incubated for 90 min at room temperature. The wells were washed with PBS/0.5% Tween-20 four times. Horseradish peroxidase (HRP)-conjugated goat anti-mouse Fab (Sigma) was then added to each well and incubated for 1 hour at room temperature. The wells were washed again four times before adding the HRP substrate 3,3',5,5'-tetramethylbenzidine (TMB, Sigma). The reaction was stopped by 2 M H₂SO₄. The absorbance of each well was read at 450 nm in a plate reader (Elx800, Bio-Tek Instruments Inc, Winooski VT).

In order to identify the methanol concentration resulting in the greatest amount of rFab expression, Clone #24-5 and Clone #5-3 were induced with methanol at various concentrations. The expression of rFab was carried out in 500 mL Erlenmeyer flasks that contained 15% (w/w, wet cell weight) of transformed *P. pichia*, i.e. Clone #24-5 and Clone #5-3, cell suspension in BMMY. Methanol was added to the culture at various concentrations (0.5%, 1%, and 1.5%) every 12 or 24 hours. Clone #24-5 and Clone #5-3 expressed the greatest amount of rFab at 48 hours after induction with 1% methanol supplemented to the culture every 12 hours.

rFab was purified from the culture medium by IMAC following ammonium sulfate precipitation. rFab was precipitated from the BMMY culture supernatant by adding ammonium sulfate to 50 % saturation at 25°C (31.3 g ammonium sulfate per 100 mL of supernatant). After overnight precipitation at 4°C, the precipitate was collected by centrifugation at 5000 × g for 20 min at room temperature, then reconstituted in 20 mM Tris-HCl buffer pH 8.0. rFab was then further purified by IMAC on a Ni-NTA agarose (Qiagen) column. Protein bound to the column was eluted with 100 mM imidazole in 20 mM Tris-HCl and 0.5 M NaCl buffer pH 8.0. The eluate was buffer-exchanged into PBS pH 7.0 using a Centricon-YM30® ultrafiltration device (Amicon, Billerica MA; M_r cut-off 30 kD). Purified rFab was characterized by SDS-PAGE, Western blot, and size-exclusion HPLC.

The yield of purified rFab from shake-flask expression was 1-2 mg/L determined by measuring the optical density at 280 nm using a molar extinction coefficient of $\epsilon = 75,560 \text{ cm}^{-1}\text{M}^{-1}$, equivalent to $E = 1.49 \text{ cm}^{-1}(\text{mg/ml})^{-1}$. The molar extinction coefficient for rFab at 280 nm was calculated (16) from the amino acid sequence of rFab derived from the reported sequence (11-14) (GenBank accession nos. L14549, L14553, V00802, and J00453).

Analysis of the Purity of rFab. The identity and purity of CC49 rFab was examined by SDS-PAGE, Western blot, and size-exclusion HPLC. For SDS-PAGE, rFab (1-2 µg) were electrophoresed under reducing (β -mercaptoethanol) and non-reducing conditions on a 4-20% Tris-HCl gradient mini-gel (Bio-Rad, Mississauga, ON), and detected by staining with Coomassie brilliant blue R-250 (Bio-Rad). For Western blot, proteins were detected by three different HRP-conjugated antibodies, i.e. goat antibodies specific for mouse Fab (Sigma), an antibody against tetra-histidine (Qiagen), and an antibody against FLAG M2® (Sigma). The antibodies were diluted at 1:2000 in PBS/0.5% Tween-20. rFab was also analyzed by size-exclusion HPLC on a BioSep® SEC-S2000 column (Phenomenex Inc., Torrance CA) eluted with 100 mM NaH₂PO₄ buffer pH 7.0 at a flow rate of 0.8 mL/min using a System Gold Model 125 HPLC interfaced with a Model 166 UV detector (Beckman-Coulter, Mississauga ON) set at 280 nm.

Coomassie brilliant blue-stained SDS-PAGE gel revealed a single band at M_r 51 kD (Fig. 3B, lane 1). This apparent molecular weight is consistent with the calculated molecular weight based on the known amino acid sequence of CC49 rFab. This band at M_r 51 kD dissociated into a band at 30 kD under reducing conditions (Fig. 3B, lane 3). Analysis by Western blot revealed that both the 51 kD and 30 kD bands were reactive with goat anti-mouse Fab antibodies (Fig. 3C, lanes 1 and 3). The 51 kD and 30 kD bands were also reactive with antibodies directed against tetra-histidine (Fig. 3E), which identifies the His₆ tag at the carboxyl terminus of the Fd chain, and also by anti-FLAG antibodies (Fig. 3F), which identifies the FLAG tag at the amino terminus of the L chain. These results confirmed that the 51 kD protein was rFab, a heterodimer, in which intermolecular disulfides covalently bridge the recombinant L and Fd chains. For comparison, SDS-PAGE analysis (Fig. 3B, lanes 2 and 4) and Western blot (Fig. 3C, lanes 2 and 4) of Fab prepared by proteolytic digestion of CC49 IgG using immobilized papain are also shown. The slightly higher M_r for rFab compared to papain-produced Fab (M_r 51 kD vs. 48 kD) was expected because the rFab included a His₆ tag, a FLAG tag, and the GGGGS peptide linker, which contribute 2.8 kD to the molecular mass. Size-exclusion HPLC (Fig. 4) showed that rFab was purified to homogeneity as indicated by a single peak with a retention time of t_R 9.2 minutes.

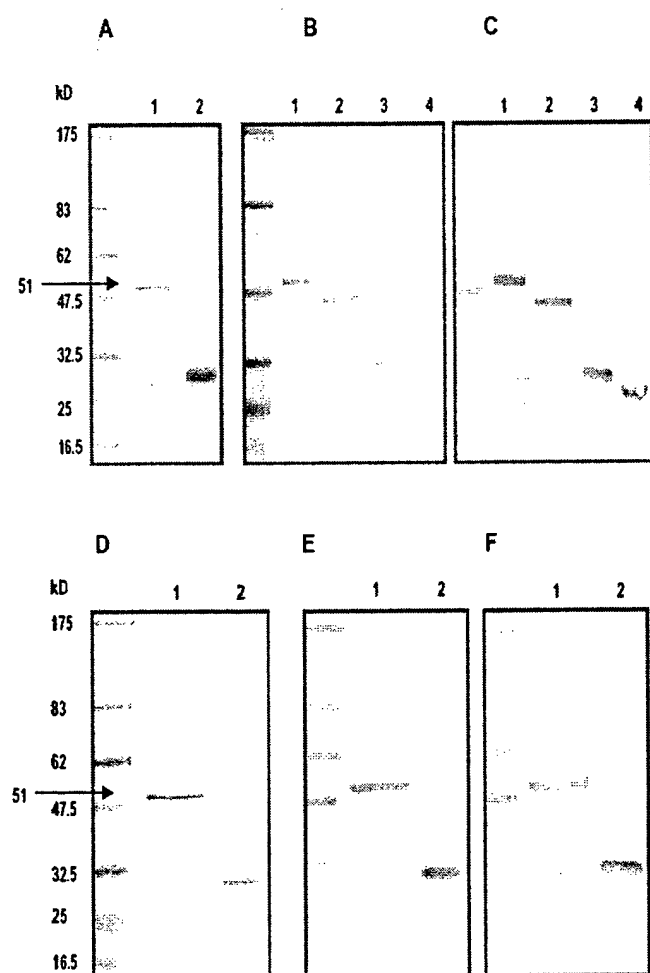


Fig. 3. SDS-PAGE and Western blot of rFab and papain-produced Fab of CC49. (A) Coomassie-stained SDS-PAGE gel of the crude culture supernatant collected at 48 h after methanol induction. The unpurified sample was concentrated 5 times and electrophoresed under non-reducing (lane 1) and reducing (β -mercaptoethanol, lane 2) conditions on a 4-20% Tris-HCl gradient gel. (B) SDS-PAGE comparison of purified rFab (lanes 1 and 3) and papain-produced Fab (lanes 2 and 4) of CC49 under non-reducing (lanes 1 and 2) and reducing (lanes 3 and 4) conditions. (C) Western blot of purified rFab (lanes 1 and 3) and papain-produced Fab (lanes 2 and 4) of CC49 under non-reducing (lanes 1 and 2) and reducing (lanes 3 and 4) conditions. The proteins were probed with a goat anti-mouse Fab. (D) Coomassie-stained SDS-PAGE gel of rFab under non-reducing (lane 1) and reducing (lane 2) conditions. (E) Western blot of purified rFab under non-reducing (lane 1) and reducing (lane 2) conditions. Proteins were probed with a tetra-histidine antibody. (F) Western blot of purified rFab under non-reducing (lane 1) and reducing (lane 2) conditions. Proteins were probed with a FLAG M2 antibody.

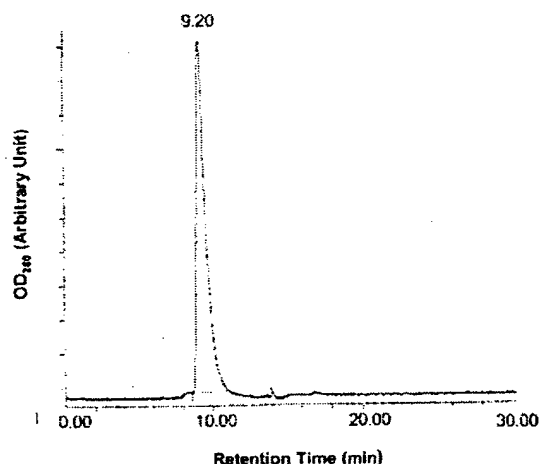


Fig. 4. Size-exclusion chromatography of purified rFab of CC49. The solid line represents UV absorbance at 280 nm. rFab was detected as a single peak with a retention time of t_R 9.2 minutes.

Task 2: Assess the immunoreactivity of the recombinant Fab

Immunoreactivity of CC49 rFab to TAG-72 *in vitro*. The *in vitro* immunoreactivity of purified rFab toward the TAG-72 antigen was assessed by ELISA. The preliminary results showed that rFab was reactive with TAG-72 in BSM immobilized on an ELISA plate. The immunoreactivity expressed as absorbance at 450 nm was plotted against molar concentrations of rFab (Fig. 5). The immunoreactivity of Fab fragments (eFab) of CC49 produced by enzymatic digestion of IgG was similarly assayed for comparison (Fig. 5). As shown, rFab was immunoreactive and the reactivity of rFab and eFab to TAG-72 in BSM increased in a concentration-dependent fashion. A more sensitive assay, such as radio-ligand binding assay, will be used to determine quantitatively the binding affinity, i.e. dissociation constant K_d , of rFab to TAG-72.

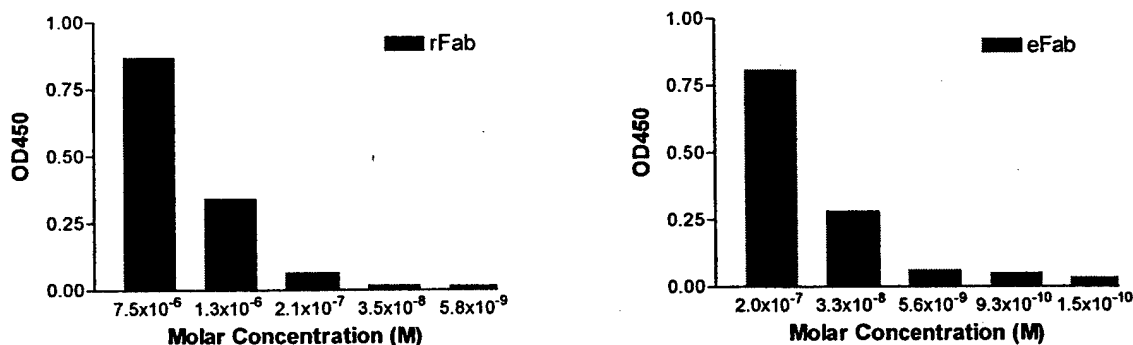


Fig. 5. ELISA assay on immunoreactivity of rFab and eFab to TAG-72 antigen present in bovine submaxillary mucin (BSM). Anti-mouse Fab horseradish peroxidase (HRP) conjugates were used to detect Fab fragments bound to TAG-72 antigen on the ELISA plate. rFab was 1:6 sequentially diluted in PBS starting at a concentration of 7.5×10^{-6} M. eFab was 1:6 sequentially diluted in PBS starting at 2.0×10^{-7} M. This concentration is 38 times lower than the starting concentration for rFab.

Task 4: Conduct in vivo imaging and biodistribution studies in a mouse tumor xenograft model

Tumor localization and biodistribution of rFab labeled with ^{123}I in athymic mice bearing TAG-72 positive s.c. LS 174T human colon cancer xenografts. Our ultimate goal is to label rFab with $^{99\text{m}}\text{Tc}$ at the hexahistidine tag fused to the amino terminus of Fd using a relatively new method (10). To demonstrate that rFab was actually immunoreactive *in vivo* with TAG-72 positive tumor in an animal model, we initially tested rFab by labeling the protein with ^{123}I using Iodogen® (Sigma) because this labeling procedure is more straightforward. The animal model was established by implanting subcutaneously 1×10^6 of LS 174T human colon cancer cells at the right flank of 4-week-old female athymic mice (Swiss *nu/nu*). 1×10^6 of A375 human melanoma cells (TAG-72 negative) were injected subcutaneously at the shoulder of the same mice as a negative control tumor. When the tumors (both LS 174T and A375 xenografts) reached a size of 2-5 mm in diameter, ^{123}I -labeled rFab was injected intravenously (tail vein) into a group of 4 mice. rFab was labeled with ^{123}I to a specific radioactivity of 0.8 $\mu\text{Ci}/\mu\text{g}$. Approximately 125 μg of ^{123}I -rFab in 100 μl PBS pH 7.0 were injected into each mouse. Twenty-four hours after injection, the mice were sacrificed and the accumulation of ^{123}I -labeled rFab in tumor and normal tissues was measured in a γ -scintillation well counter with a 40 keV window around the 159 keV photopeak. Tumor uptake (percent injected dose per gram, % ID/g) and tumor-to-normal tissue (T/NT) ratios were analyzed (Fig. 6).

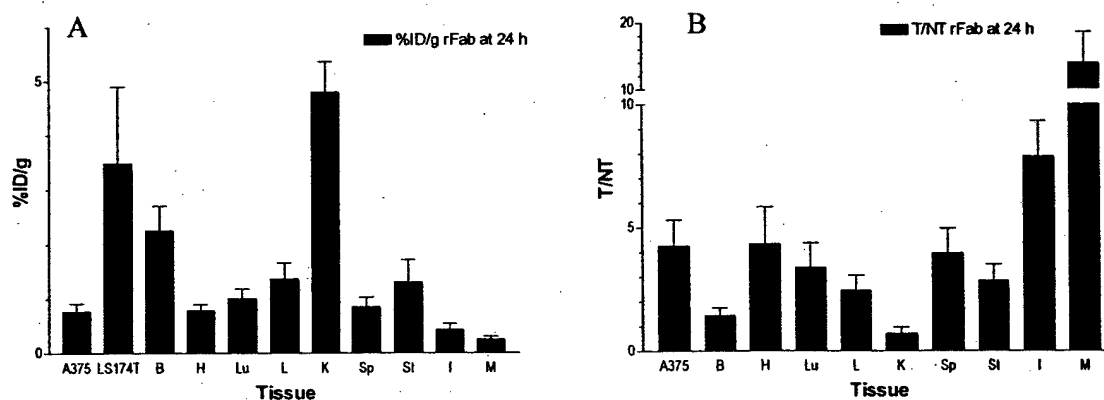


Fig. 6. (A) Uptake in tissues at 24 h after administration of ^{123}I -rFab. **(B)** T/NT ratios at 24 h. T/NT ratios were calculated by the dividing uptake in TAG-72 positive LS 174T tumor by the uptake in normal tissues or TAG-72 negative tumor A375 (negative control). Tissues shown are B (blood), H (heart), Lu (lungs), L (liver), K (kidneys), Sp (spleen), St (stomach), I (intestine), and M (muscle).

Tumor uptake (Fig. 6A) of ^{123}I -rFab at 24 h post-injection in LS 174T xenografts was 3.5 ± 1.40 % ID/g. Tumor uptake was only 0.8 ± 0.144 % ID/g in TAG-72 negative A375 xenografts (one-tailed *t* test, $p = 0.056$). Uptake in the blood was 2.3 ± 0.45 % ID/g. Kidneys showed the highest uptake, while muscle showed the lowest (4.8 ± 0.56 vs. 0.25 ± 0.053 % ID/g). The uptake ratio of LS 174T to A375 (Fig. 6B) was 4.3 ± 1.06 . T/B ratio was 1.4 ± 0.31 . The highest T/NT ratio occurred in muscle at 13.9 ± 4.61 . These preliminary results suggest that ^{123}I -rFab localized in TAG-72 positive LS 174T tumor, but not in TAG-72 negative A375 tumor, possibly through the mediation of TAG-72 antigen. The biodistribution profile of ^{123}I -rFab resembled that of typical small antibody fragments with a characteristically high kidney uptake. Labeling with $^{99\text{m}}\text{Tc}$ through the hexahistidine tag in rFab will be

tested in Year 2. An animal study using ^{99m}Tc labeled rFab will be conducted to investigate tumor uptake and biodistribution of the radiopharmaceutical.

PLANNED RESEARCH FOR YEAR 2 (2004-2005)

Task 2 (completion): Assess the immunoreactivity of the recombinant Fab. In the second year of the project, I shall complete the assessment of the immunoreactivity of rFab to the TAG-72 antigen using an ELISA method or a competition radioligand binding assay. The antigen-binding activity of rFab will be compared with that of enzymatically generated Fab by papain. I may also test the immunoreactivity of rFab with surgical specimens from DCIS using immunohistochemical staining.

Task 3: Label recombinant Fab with ^{99m}Tc -tricarbonyl complex through the carboxyl-terminal His₆ tag. Labeling a recombinant protein with ^{99m}Tc -tricarbonyl complex through a terminal His₆ tag is a relatively new method, therefore, I initially plan to use a synthetic polypeptide to work out labeling conditions. A peptide (GGGGSHHHHH) has been synthesized to contain the sequence at the carboxyl terminus of Fd in CC49 rFab. I have also obtained the IsoLinkTM carbonyl labeling agent (a 10 mL sealed vial contains lyophilized formulation of 8.5 mg sodium tartrate, 2.85 mg of sodium tetraborate, 7.15 mg of sodium carbonate, and 4.5 mg of sodium boranocarbonate; Mallinckrodt Medical B.V., the Netherlands). The chemical agent allows a single-step preparation of $[\text{}^{99m}\text{Tc}(\text{CO})_3(\text{OH})_2]^{+}$, the complex used to form stable coordinate covalent bonds with a terminal His₆ in a protein. Using the peptide as a model, I shall examine how various reaction conditions, such as pH, temperature, and the incubation time, may affect the radiolabeling efficiency and specificity. The reaction conditions will later be customized for labeling CC49 rFab with ^{99m}Tc to achieve a high radiolabeling efficiency and specificity.

Task 4 : Conduct in vivo imaging and biodistribution studies in a mouse tumor xenograft model. In a preliminary study, rFab of CC49 labeled with ^{123}I has demonstrated specific localization in TAG-72 positive LS174T human colon cancer xenografts subcutaneously implanted in athymic mice. The biodistribution of radioactivity in tumor and normal tissues of these mice, however, was only examined at 24 hours post-injection. In the second year of this project, I plan to examine biodistribution of rFab labeled with ^{99m}Tc or ^{123}I at 2, 6 and 24 hours after i.v. injection of the radiolabeled rFab into the tumor-bearing mice. The mice will also be imaged using a small-field-of-view γ -camera.

Task 5: Analyze data statistically and prepare manuscripts. I shall analyze the data from the *in vitro* and *in vivo* studies and prepare manuscripts for submission to a peer-reviewed scientific journal. In second year of this project, I shall complete the writing of my PhD thesis and defend it.

KEY RESEARCH ACCOMPLISHMENTS

- Cloned the genes for the light chain and Fd portion of the heavy chain of the monoclonal antibody CC49 from hybridoma cells.
- Constructed co-expression plasmids for expression of a recombinant Fab of CC49 containing an integrated radiometal-binding site for ^{99m}Tc .

- Expressed the recombinant Fab in *Pichia pastoris*, purified the recombinant Fab, and characterized its purity.
- Characterized the immunoreactivity of the recombinant Fab to the TAG-72 antigen *in vitro* and in an animal xenograft model in preliminary studies.

REPORTABLE OUTCOMES

Manuscripts

Ying Tang, Shaoxian Yang, Jean Gariepy, and Raymond M. Reilly. Cloning and Expression of Recombinant Fab (rFab) Fragments of the Tumor-Associated Glycoprotein-72 (TAG-72) Monoclonal Antibody CC49 in *Pichia pastoris*. Manuscript in preparation, 2004.

Ying Tang, Judy Wang, Deborah Scollard, Claire Holloway, Hridya Mondal, Harriette J. Kahn and Raymond M. Reilly. Preparation, characterization and evaluation of ¹¹¹In-labeled trastuzumab (Herceptin®) Fab fragments in athymic mice bearing HER-2/neu positive BT-474 human breast cancer xenografts. Submitted, 2004.

Abstracts

Ying Tang (presenter), Shaoxian Yang, Jean Gariepy, Raymond M. Reilly. Optimized Expression of rFab of the TAG-72 Monoclonal Antibody CC49 in *Pichia pastoris* and Preliminary Evaluation of Its Tumor Targeting Properties in the LS174T Human Colon Cancer Xenograft Model. Proc. Amer. Assoc. Cancer Res. 45:165 [abstract 714], 2004.

Presentations

Ying Tang. Construction of Recombinant Fab Fragments of Monoclonal Antibody CC49 for Radioimmunoguided Surgery of Breast Cancer. Graduate Research in Progress Symposium, Dept. of Pharmaceutical Sciences, University of Toronto, February 12, 2003.

CONCLUSIONS

In conclusion, a recombinant Fab (rFab) of monoclonal antibody CC49 was successfully expressed in *Pichia pastoris* at a yield of 1-2 mg/L. rFab of CC49 was purified to homogeneity. rFab showed immunoreactivity *in vitro* with the TAG-72 antigen in bovine submaxillary mucin using ELISA. An *in vivo* study showed that rFab labeled with ¹²³I specifically localized in TAG-72 positive LS174T tumor xenografts subcutaneously implanted in athymic mice. These results warrant further investigation of this rFab labeled with ^{99m}Tc through the integrated carboxyl-terminal His₆ tag as a potential radiopharmaceutical for radioimmunoguided surgery of DCIS.

REFERENCES

1. Peeters, P. H., Verbeek, A. L., Straatman, H., Holland, R., Hendriks, J. H., Mravunac, M., Rothengatter, C., Dijk-Milatz, A., and Werre, J. M. Evaluation of overdiagnosis of breast cancer in screening with mammography: results of the Nijmegen programme. *Int. J. Epidemiol.*, **18**: 295-299, 1989.
2. Baker, L. H. Breast Cancer Detection Demonstration Project: five-year summary report. *CA Cancer J. Clin.*, **32**: 194-225, 1982.
3. Lagios, M. D. and Silverstein, M. J. Ductal carcinoma in situ. The success of breast conservation therapy: a shared experience of two single institutional nonrandomized prospective studies. *Surg. Oncol. Clin. N. Am.*, **6**: 385-392, 1997.
4. Goldenberg, D. M. Future role of radiolabeled monoclonal antibodies in oncological diagnosis and therapy. *Semin. Nucl. Med.*, **19**: 332-339, 1989.
5. Hetelekidis, S., Schnitt, S. J., Morrow, M., and Harris, J. R. Management of ductal carcinoma in situ. *CA Cancer J. Clin.*, **45**: 244-253, 1995.
6. Lagios, M. D. Duct carcinoma in situ. Pathology and treatment. *Surg. Clin. North Am.*, **70**: 853-871, 1990.
7. Frykberg, E. R. and Bland, K. I. Overview of the biology and management of ductal carcinoma in situ of the breast. *Cancer*, **74**: 350-361, 1994.
8. Lagios, M. D., Westdahl, P. R., Margolin, F. R., and Rose, M. R. Duct carcinoma in situ. Relationship of extent of noninvasive disease to the frequency of occult invasion, multicentricity, lymph node metastases, and short-term treatment failures. *Cancer*, **50**: 1309-1314, 1982.
9. Contegiacomo, A., Alimandi, M., Muraro, R., Pizzi, C., Calderopoli, R., De Marchis, L., et al. Expression of epitopes of the tumour-associated glycoprotein 72 and clinicopathological correlations in mammary carcinomas. *Eur. J. Cancer*, **30A**: 813-820, 1994.
10. Waibel, R., Alberto, R., Willuda, J., Finnern, R., Schibli, R., Stichelberger, A., Egli, A., Abram, U., Mach, J. P., Pluckthun, A., and Schubiger, P. A. Stable one-step technetium-99m labeling of His-tagged recombinant proteins with a novel Tc(I)-carbonyl complex. *Nat. Biotechnol.*, **17**: 897-901, 1999.
11. Sakano, H., Rogers, J. H., Huppi, K., Brack, C., Traunecker, A., Maki, R., Wall, R., and Tonegawa, S. Domains and the hinge region of an immunoglobulin heavy chain are encoded in separate DNA segments. *Nature*, **277**: 627-633, 1979.
12. Honjo, T., Obata, M., Yamawaki-Katoaka, Y., Kataoka, T., Kawakami, T., Takahashi, N., and Mano, Y. Cloning and complete nucleotide sequence of mouse immunoglobulin gamma 1 chain gene. *Cell*, **18**: 559-568, 1979.
13. Hamlyn, P. H., Brownlee, G. G., Cheng, C. C., Gait, M. J., and Milstein, C. Complete sequence of constant and 3' noncoding regions of an immunoglobulin mRNA using the dideoxynucleotide method of RNA sequencing. *Cell*, **15**: 1067-1075, 1978.
14. Rixon, M. W., Gourlie, B. B., Kaplan, D. A., Schlom, J., and Mezes, P. S. Preferential use of a H chain V region in antitumor-associated glycoprotein-72 monoclonal antibodies. *J. Immunol.*, **151**: 6559-6568, 1993.
15. Gonzales, N. R., Padlan, E. A., De Pascalis, R., Schuck, P., Schlom, J., and Kashmiri, S. V. Minimizing immunogenicity of the SDR-grafted humanized antibody CC49 by genetic manipulation of the framework residues. *Mol. Immunol.*, **40**: 337-349, 2003.
16. Gill, S. C. and von Hippel, P. H. Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.*, **182**: 319-326, 1989.